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Report

ARF GEF-Dependent Transcytosis and Polar Delivery of PIN Auxin Carriers in *Arabidopsis*

Jürgen Kleine-Vehn,[1,2] Pankaj Dhonukshe,[2,4] Michael Sauer,[1,2] Philip B. Brewer,[2,5] Justyna Wiśniewska,[2,6] Tomasz Paciorek,[2,7] Eva Benková,[1,2] and Jiří Friml[1,2,3,*]

[1]Department of Plant Systems Biology
Flanders Institute for Biotechnology (VIB)
and Department of Molecular Genetics
Ghent University
9052 Gent
Belgium

[2]Zentrum für Molekularbiologie der Pflanzen (ZMBP)
Universität Tübingen
72076 Tübingen
Germany

[3]Albrecht-von-Haller-Institut für Pflanzenwissenschaften
Universität Göttingen
37073 Göttingen
Germany

Summary

Cell polarity manifested by the polar cargo delivery to different plasma-membrane domains is a fundamental feature of multicellular organisms. Pathways for polar delivery have been identified in animals; prominent among them is transcytosis, which involves cargo movement between different sides of the cell [1]. PIN transporters are prominent polar cargoes in plants, whose polar subcellular localization determines the directional flow of the signaling molecule auxin [2, 3]. In this study, we address the cellular mechanisms of PIN polar targeting and dynamic polarity changes. We show that apical and basal PIN targeting pathways are interconnected but molecularly distinct by means of ARF GEF vesicle-trafficking regulators. Pharmacological or genetic interference with the *Arabidopsis* ARF GEF GNOM leads specifically to apicalization of basal cargoes such as PIN1. We visualize the translocation of PIN proteins between the opposite sides of polarized cells in vivo and show that this PIN transcytosis occurs by endocytic recycling and alternative recruitment of the same cargo molecules by apical and basal targeting machineries. Our data suggest that an ARF GEF-dependent transcytosis-like mechanism is operational in plants and provides a plausible mechanism to trigger changes in PIN polarity and hence auxin fluxes during embryogenesis and organogenesis.

*Correspondence: jiri.friml@psb.ugent.be
[4]Present address: Department of Biology, Utrecht University, 3508 TB Utrecht, The Netherlands.
[5]Present address: Centre for Integrative Legume Research, University of Queensland, Brisbane, Australia.
[6]Present address: Institute of General and Molecular Biology, Nicolas Copernicus University, 87-100 Toruń, Poland.
[7]Present address: Department of Biological Sciences, University of California, Stanford, California 94305-5020, USA.

Results and Discussion

PIN Targeting Utilizes Distinct ARF GEF-Dependent Apical and Basal Pathways

Previous studies have suggested that the polar delivery of auxin efflux and influx components occurs via different pathways [4, 5]. The fungal toxin brefeldin A (BFA) has been shown to specifically inhibit a subclass of ARF GEFs. The recycling of PIN auxin efflux carriers from endosomes to the plasma membrane is BFA sensitive, as reflected by the accumulation of PINs in aggregated endosomal BFA compartments in response to BFA [6, 7].

We observed that the localization of PIN proteins at the apical (shoot-apex-facing) and basal (root-apex-facing) sides of *Arabidopsis* root cells differed dramatically in their sensitivity to the BFA treatment. Within 60 min, basal cargoes, such as PIN1 in the stele or PIN2 in the young cortex cells, were almost completely internalized from the plasma membrane into BFA compartments, whereas most of the apically localized PIN2 in the epidermis remained at the plasma membrane in addition to its intracellular accumulation (Figures 1A–1D). To address whether apical and basal targeting display differential sensitivities to BFA in the same cell type, we used transgenic lines to target two different, but both functional, versions of PIN1 to opposite sides of epidermal cells [2]. After short-term BFA treatments, the basal PIN1-HA (Figure 1E) was almost completely internalized within 60 min of BFA treatment (Figure 1F), whereas the apical PIN1-GFP-3 remained to a large degree at the apical plasma membrane (Figures 1G and 1H). Preferential BFA-induced internalization of the basal cargoes was particularly apparent when PIN1-HA (basal) and endogenous PIN2 (apical) were simultaneously visualized in the same epidermal cells (Figures 1I and 1J).

These experiments show that the apical and basal targeting in plants involves different sets of ARF GEF proteins. Basal targeting strictly requires BFA-sensitive ARF GEFs, whereas apical delivery is largely insensitive to BFA. Consequently, apical and basal pathways in plants are distinct and operate in parallel in the same cell.

Inhibition of BFA-Sensitive ARF GEFs Leads to Recruitment of Basal PIN Cargoes into the Apical Pathway

Next, we addressed whether the apical and basal targeting pathways are interconnected and whether they can alternatively be used by the same cargoes. The inhibitory effect of BFA was exploited to interfere preferentially with basal, but not apical, PIN targeting.

After prolonged BFA treatments, the internalized PIN1 in BFA compartments decreased and PIN1 gradually appeared at the apical plasma membrane (Figures 2A–2C). For PIN2 in the cortex cells, BFA incubations as short as 2 hr were sufficient to induce a pronounced basal-to-apical polarity shift (Figure 2D). Longer treatments or the use of higher BFA concentrations confined the localization of both PIN1 and PIN2 to a restricted region in the middle of the apical membrane, designated "superapical domain" (Figures 2C and 2E). In contrast to basal cargoes, apical cargoes, such as PIN2 or PIN1-GFP-3, in the epidermis stayed at the apical plasma membrane

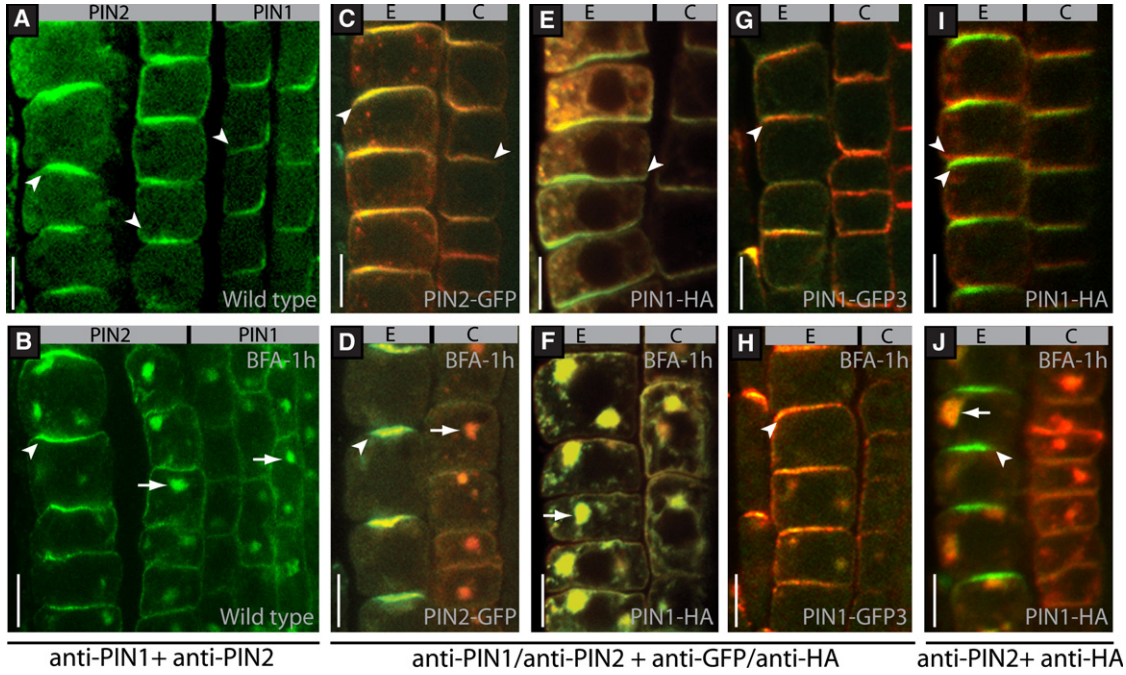

Figure 1. Distinct Apical and Basal ARF GEF-Dependent Targeting of PIN Proteins

(A and B) Apical localization of PIN2 in the epidermis; basal localization of PIN2 in the cortex and PIN1 in the stele and endodermis cells (A). BFA treatment (50 μM) leads to a strong internalization of the basal PIN1 and PIN2 but not apically localized PIN2 (B).

(C and D) Apical localization in the epidermis, but basal in the cortex cells of PIN2-GFP (C). In cortex cells, basal PIN2-GFP rapidly internalizes after BFA incubation, whereas in epidermal cells it displays BFA-resistant plasma-membrane localization (D).

(E and F) Basal localization in epidermal and cortex cells of PIN1-HA (E). Basally localized PIN1-HA shows a rapid BFA-dependent internalization in both cell types (F).

(G and H) PIN2-like distribution in epidermal (apical) and cortex (basal) cells of PIN1-GFP-3 (G). Apical PIN1-GFP-3 localization in the epidermis is resistant to BFA treatment (H).

(I and J) Simultaneous visualization of apically localized PIN2 (green) and basal PIN1-HA (red) in epidermal cells (I); PIN1-HA internalizes completely after BFA treatment, whereas PIN2 largely remains at the apical plasma membrane (J).

Arrows depict PIN proteins in the BFA compartments and arrowheads PIN polarity. E denotes epidermis; C denotes cortex. Scale bars represent 10 μm.

even after prolonged BFA treatment (Figures 1H and 1J; Figure S1E available online). Notably, these effects were specific for polar cargoes (Figures S1A and S1B) and were not based on a mere disappearance of the BFA compartments (Figures S1C–S1E).

Recently, it has been shown that *Arabidopsis* Sorting Nexin1 (SNX1)-dependent and Vacuolar Protein Sorting 29 (VPS29)-dependent pathways are involved in PIN targeting [8, 9]. However, BFA-induced PIN apicalization was still observed in corresponding *snx1* and *vps29* mutants (Figures S2A–S2C); this result does not support a role of these regulators at the intersection of ARF GEF-dependent apical and basal pathways.

Taken together, these data provide the mechanistic insight that inhibition of BFA-sensitive ARF GEFs leads to a recruitment of basal cargoes into the apical targeting pathway. This suggests that the apical and basal targeting pathways are interconnected and can be used alternatively by the same polar cargoes.

Inhibition of ARF GEF GNOM Is Sufficient to Recruit Basal PIN Cargoes into the Apical Pathway

Next we addressed which BFA-sensitive ARF GEFs are required for the BFA-induced basal-to-apical shift in PIN polarity. The endosomal ARF GEF GNOM was an obvious candidate because GNOM is sensitive to BFA and has been shown to be involved in the endosome-to-plasma membrane targeting of different cargoes, including PIN1 [7].

We used transgenic lines in which the wild-type GNOM had been replaced by an engineered BFA-resistant version (*GNOM*^{M696L}) [7]. In these lines, BFA-dependent apicalization of PIN1 or PIN2 in cortex cells did not occur (Figures 2F and 2G), showing that it is the specific inhibition of GNOM by BFA that leads to the BFA-induced PIN apicalization. Next, we examined partial loss-of-function alleles of *GNOM* (*gnom*^{R5} and *van7*) that initially form correctly patterned roots after germination [10, 11]. Basal cargoes, such as PIN1 in the stele and PIN2 in the cortex cells, showed apicalization reminiscent of long-term BFA treatment (Figures 2H and 2I). On the other hand, the localization of apical cargoes, such as PIN2 in epidermal cells, and nonpolar cargoes, such as plasma membrane H^+-ATPase, were unaffected in either of these *gnom* alleles (Figure 2I; data not shown). In analogy to BFA treatments, we observed cells with "superapical" PIN localization in untreated *gnom* alleles (Figure 2I). Notably, BFA treatments led to a reduced PIN accumulation in BFA compartments and to a faster and more pronounced PIN apicalization in *gnom* mutant roots compared to wild-type roots (Figures S3A and S3B).

These results show that pharmacological or genetic inhibition of the GNOM ARF GEF is sufficient to recruit basal cargoes into the apical pathway. In addition to its general function in endosome-to-plasma membrane trafficking for polar and nonpolar cargoes [7, 12], GNOM obviously plays a role specifically in basal targeting, whereas apical targeting is independent of GNOM and possibly requires BFA-resistant ARF GEFs.

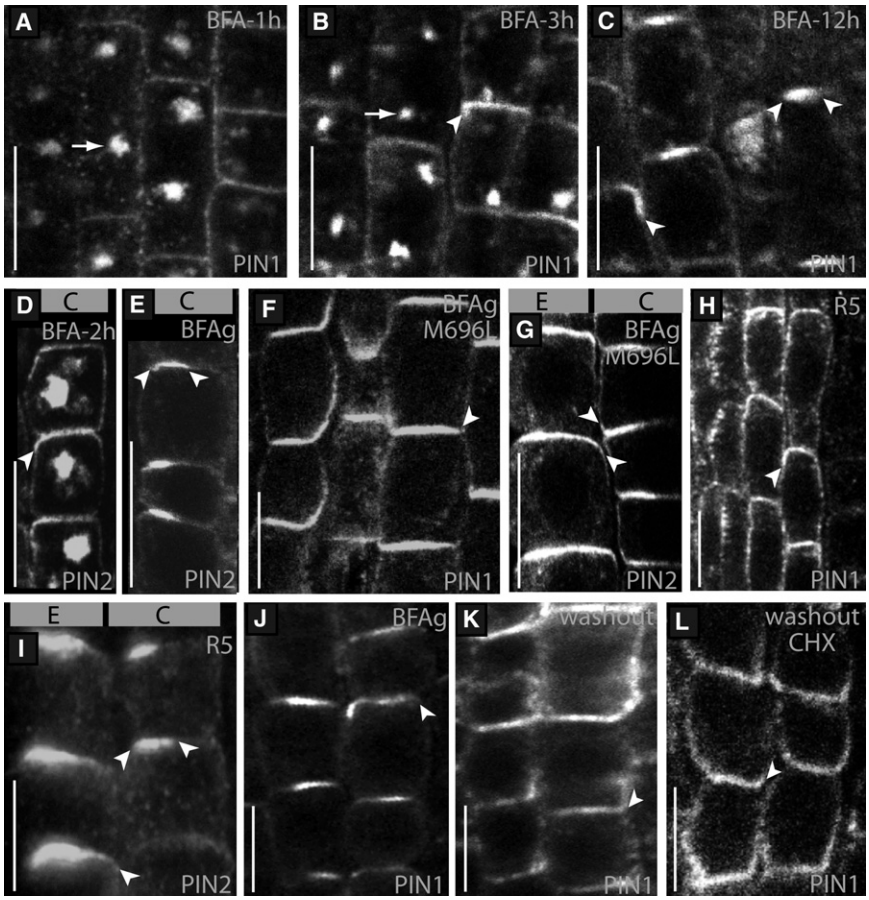

Figure 2. Apicalization of Basal Cargoes after Inhibition of BFA-Sensitive ARF GEF GNOM

(A–C) PIN1 targeting after BFA (50 μM) treatment. In the stele, PIN1 almost completely internalizes after 1 hr (A). Prolonged BFA incubation (2–3 hr) leads to a decrease of PIN1 in BFA compartments and its gradual occurrence at the apical plasma membrane (B). After 12 hr of BFA treatment, PIN1 is absent in the BFA compartment and completely confined to the apical plasma membrane (C).

(D and E) Short-term BFA treatment (50 μM; 2 hr) results in a basal-to-apical shift of PIN2 in cortex cells (D), whereas BFA (25 μM)-germinated seedlings display super apical PIN2 localization in cortex cells (E).

(F and G) The engineered BFA-resistant *GNOM*^{M696L} seedlings do not show the BFA-induced basal-to-apical shift of PIN1 in the stele (F) and of PIN2 in the cortex cells (G) even after several days of BFA exposure.

(H and I) Basal-to-apical shift of PIN1 (H) and PIN2 (I) in untreated root cells of partial loss-of-function *gnom*^{R5} mutant.

(J–L) Apical localization of PIN1 in wild-type seedlings germinated on BFA (J). Translocation of PIN1 to the basal plasma membrane after BFA removal (2 hr in liquid MS) (K) and in the presence of the protein-biosynthesis inhibitor cycloheximide (50 μM; 2 hr) (L).

Immunocytochemistry of anti-PIN1 (A–C, F, H, J–L) and anti-PIN2 (D, E, G, I); arrowheads depict polar localization of PIN proteins. E denotes epidermis; C denotes cortex. Scale bars represent 10 μm.

Inhibition of ARF GEF GNOM Reveals Directional Transcytosis of PIN Proteins between Apical and Basal Polar Domains

The BFA-induced basal-to-apical shift of PIN proteins was fully reversible, and the re-establishment of basal PIN localization after BFA removal was independent of protein biosynthesis (Figures 2J–2L). Moreover, fluorescent recovery after photobleaching (FRAP) of PIN2-GFP at the basal cell side after BFA removal depended on the apical pool of PIN2-GFP (Figure 3A; Figure S4A). These results suggest that the same cargo molecules were translocating between apical and basal cell sides.

To test this scenario directly, we used the green-to-red photoconversion capability of the fluorescent tag EosFP [13], enabling us to trace the dynamics of PIN2-EosFP in vivo. In *35S:PIN2-EosFP*-overexpressing lines, PIN2-EosFP localized strongly to the apical as well as, to some extent, to the basal side of epidermal cells [13]. We observed internalization of formerly basally localized PIN2-EosFP after BFA treatment and its subsequent appearance at the upper cell periphery, demonstrating PIN2 translocation from the basal to the apical cell side (Figure 3B). In polarized cells, BFA treatment typically leads to formation of two BFA compartments per cell positioned closer to either the apical or basal side of the cell [6]. We observed a rapid translocation of PIN2-EosFP from the basal to the apical compartment (Figures 3C and 3D). In contrast, PIN2-EosFP from the apical compartment translocated predominantly to the apical plasma membrane and never to the basal BFA compartment (Figures 3C and 3D). As expected, this basal-to-apical shift of PIN2-EosFP was compromised in the *GNOM*^{M696L} line expressing the BFA-resistant GNOM version (Figures S4B and S4C). Thus, both the PIN2-GFP FRAP and PIN2-EosFP photoconversion in the presence of BFA or after its removal reveal unidirectional translocation of PIN2 molecules between apical and basal cell sides.

Next we investigated whether dynamic translocation of PIN protein in root epidermal cells also occurs independently of BFA treatments. In our experimental conditions, it was difficult to visualize translocation of activated PIN2-EosFP in interphase cells (Figures S4D and S4E). However, we detected substantial retargeting of PIN2-EosFP from the apical and basal cell sides to the newly completed plasma membrane after cell division (Figures 3E and 3F, and Figures S5A and S5B). This observation extends on the recent finding on the importance of sterol-dependent endocytosis for PIN2 polarity re-establishment after cytokinesis [14] and confirms our hypothesis that dynamic translocation of PIN proteins between apical and basal cell sides occurs in vivo.

In animal cells, movement of cargoes, such as the transferrin receptor, between separated plasma-membrane domains is termed transcytosis [15]. Because our experiments provide an in vivo demonstration of a comparable event in plant cells, we propose the use of the same term. In further analogy to animal cells [16, 17], our findings reveal that the apical-to-basal transcytosis of PIN proteins involve BFA-sensitive ARF GEFs. Thus, basal targeting of PIN proteins in polarized plant cells and the basolateral localization of transferrin receptors in animal cells are remarkably analogous and might possibly follow an evolutionarily conserved principle.

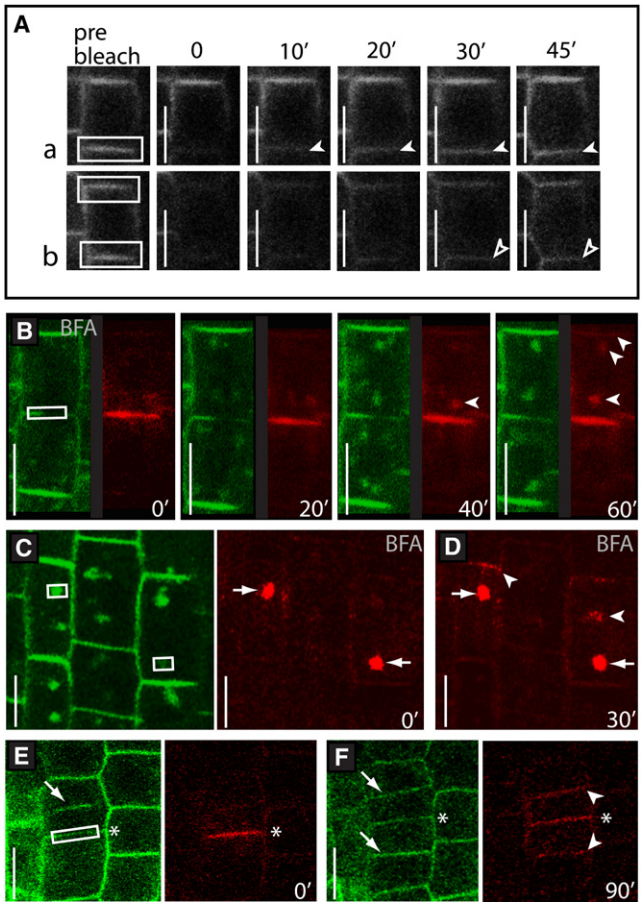

Figure 3. BFA-Dependent Apical-Basal Transcytosis of PIN2

(A) FRAP experiments in the presence of cycloheximide (50 μM; 45 min pretreatment) illustrating the apical-to-basal transcytosis of PIN2 in cortex cells. After long-term BFA treatment (50 μM; 12 hr), apicalized PIN2-GFP in the cortex cells rapidly recovers at the basal plasma membrane following BFA removal (A_a). This recovery is compromised after additional photobleaching of the apical pool of PIN2 GFP (A_b).

(B) Representative pictures illustrating time lapse of PIN2-EosFP (green) and photoconverted (red) in the presence of BFA (50 μM) 0, 20, 40, and 60 min after photoconversion. Gradual translocation of photoconverted PIN2-EosFP from the basal to the apical cell sides through the BFA compartments.

(C and D) BFA (50 μM; 1 hr)-treated PIN2-EosFP-expressing cells (green) 0 (C) and 30 min (D) after photoconversion (red). Photoconverted PIN2-EosFP translocated from the BFA compartment to the apical cell side or from the basally localized BFA compartment to the apically localized compartment.

(E and F) PIN2-EosFP (green) and activated PIN2-EosFP (red) shortly (E) and 90 min (F) after activation in untreated root epidermal cells. Freshly divided cells show substantial PIN2 translocation to the newly built cell sides (F).

Live-cell imaging of PIN2-FP (A–F); arrows indicate activated BFA compartments (C and D) or freshly divided cells (E and F), asterisks depict activated cell side, and arrowheads the translocated PIN2-FP. Scale bars represent 10 μm.

GNOM Mediates the Dynamic Establishment of Basal PIN Polarity during Embryogenesis and Postembryonic Organogenesis

Our data suggest that a GNOM-dependent transcytosis mechanism can be utilized to re-establish basal PIN localization in polarized cells. In addition to a set PIN polar localization in different cell types [18–20], basal PIN1 localization is established dynamically during various developmental processes including formation of embryonic axes [21], organogenesis [11, 22], and formation of vasculature [23, 24]. All these events also require GNOM, which prompted us to re-examine selected developmental events from a transcytosis point of view.

The earliest known rearrangement of PIN polarity is the switch from apolar to basal localization of PIN1 in the inner cells of the *Arabidopsis* 32-cell-stage proembryo; this switch establishes directional auxin flow to the region of the future root meristem [21]. We inhibited ARF GEF function at relevant stages by using in vitro cultures of *Arabidopsis* embryos within their ovules [25]. Embryos that were cultivated in the presence of BFA failed to polarize PIN1 to the basal side of provascular cells, but preferentially showed PIN1 at the apical cell side (Figure 4B). Analogous to roots, apical or nonpolar cargoes remained unaffected (Figure 4B; data not shown). In contrast, in BFA-resistant *GNOM*^{M696L} embryos, PIN1 polarized normally before the 64-cell stage in the presence of BFA (Figure 4C). Consistently with these observations, the earliest PIN1 polarity defect in *gnom* mutant embryos is the failure of early PIN1 polarization to the basal sides of provasculature cells, whereas the apical PIN1 localization in outer layers remained largely unaffected (Figures 4D and 4E). In line with the role of PIN proteins in directional auxin distribution [2], apicalization of PIN1 during embryogenesis affected the auxin distribution and apical-basal embryo patterning (Figures S6A–S6D). Our data substantiate previous findings [21, 26] on the role of GNOM ARF GEF in embryonic patterning and suggest that the initial function of GNOM in embryogenesis is the early establishment of basal polarization of PIN1 in provascular cells.

Similar to the situation in embryogenesis, the development of various organ primordia is accompanied by dynamic rearrangements of PIN polarity [22, 27, 28]. In lateral root primordia, PIN1 localization undergoes a polarity switch from the anticlinal to the outer periclinal cell side that represents the new basal (lateral-root-apex-facing) side [11, 22]. This polarity switch of PIN1 occurs early between stages I and III of primordium development (Figure S6E). Pharmacological [22] or genetic [11] inhibition of the ARF GEF function interferes with PIN1 polar localization during primordia formation and, consequently, with their development. We observed that inhibition of the ARF GEF function by BFA did not primarily alter the advent of PIN1 polarity shift to the periclinal side, but that PIN1 localized predominantly to the opposite, inner periclinal cell side (Figures 4F and 4G). In contrast, no defects in PIN1 relocation or auxin distribution were observed in the BFA-resistant *GNOM*^{M696L} line after BFA incubation (Figures 4J and 4K). These results support the assumption that GNOM mediates dynamic PIN polarity changes to the outer periclinal cell side, which are required for the establishment of new growth axes during de novo organ formation. Notably, this polarization event defines the new basal cell side of the future organ.

Collectively, our findings show that GNOM is required for switches of PIN polarity to the basal cell side that are occurring during embryonic and in postembryonic organ formation. Evident analogies between these events and the transcytosis-like mechanism detected in polarized root cells suggest that these developmental events are mediated by a GNOM-dependent transcytosis mechanism.

Conclusions

Our data provide novel insights into the mechanism of dynamic subcellular polar delivery of PIN auxin efflux carriers and thus into the control of intercellular auxin flow. We show for the first time that cargoes exemplified by PIN proteins

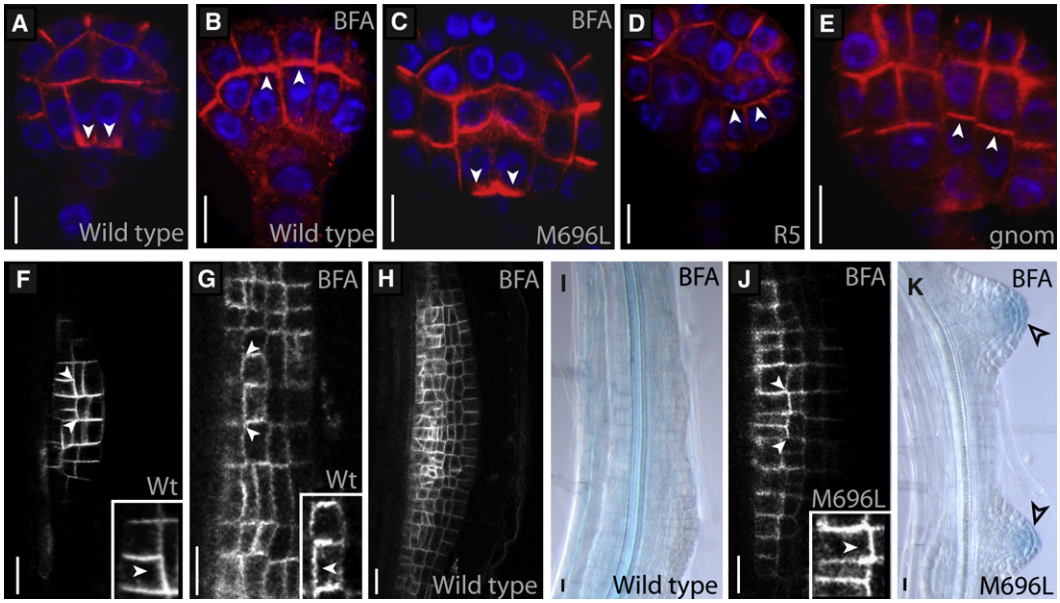

Figure 4. ARF GEF GNOM-Dependent Basal Retargeting of PIN1 in Embryogenesis and Lateral Root Organogenesis

(A–E) PIN1 polarization during apical-basal axis formation. Basal polarization of PIN1 in provascular cells at the 32-cell stage of the *Arabidopsis* embryos (A). BFA-treated (10 μM; 7 days) embryos fail to correctly polarize PIN1 (B). PIN1 translocation to the basal plasma membrane in BFA-resistant $GNOM^{M696L}$ mutants after BFA treatments is shown (C). Partial loss-of-function $gnom^{R5}$ mutant embryos display defects in the basal targeting of PIN1 (D). Full-knockout *gnom* embryos show apical PIN1 localization in all cell types (E).

(F–K) Establishment of an anticlinal-to-periclinal shift in PIN1-GFP localization, pointing toward the presumptive primordium tip during lateral root development (F). BFA-treated (10 μM; 48 hr) roots display predominant inward localization (away from the primordium tip) (G). Unorganized cell division and loss of cellular polarity during progression of lateral root primordia (H) as well as altered auxin response (*DR5:GUS*) distribution (I) after BFA treatments are shown. PIN1-GFP polarized toward the primordia tip after BFA treatment in $GNOM^{M696L}$ (J), resulting in rescued *DR5:GUS* pattern (K).

Immunocytochemical images (A–E) and live-cell imaging of lateral root primordia (F–H and J). Open and filled arrowheads indicate DR5 activity and polar localization of PIN1, respectively. Scale bars represent 10 μm.

can move between different sides of plant cells and by this means change their polar localization. This transcytosis mechanism is realized by a combination of constitutive endocytic recycling [6, 13, 29] and alternative recruitment of cargoes by distinct ARF GEF-dependent apical and basal targeting machineries (Figure S7). The endosomal ARF GEF GNOM plays in this context a specific role in basal recycling. It seems that maintenance of the basal PIN localization in polarized root cells and its dynamic establishment in young embryos or lateral root primordia share a common GNOM-dependent polar-targeting mechanism. In this scenario, the evolutionarily conserved mechanism of GNOM-dependent PIN transcytosis to the basal cell side (Figure S7) would be utilized in plants for a wide range of developmental processes involving rapid changes in PIN polarity. Diverse developmental or environmental [30] cues could thus be integrated to redirect the auxin flow between cells, altogether modulating patterns of auxin-dependent development.

Supplemental Data

Experimental Procedures and seven figures are available at http://www.current-biology.com/cgi/content/full/18/7/526/DC1/.

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